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An Inhibitor Protein of the wnt Signal Path

The present invention relates to an inhibitor protein of the wnt signal path, a DNA encoding such a protein, and a process for preparing such a protein. Furthermore, this invention concerns the use of the DNA and the protein as well as antibodies directed against the protein.

The wnt signal path plays an important part for the regulation of cell proliferation and differentiation during the embryonal development of Drosophila, *Xenopus laevis* and mice. The wnt signal path comprises the combination of secretory glycoproteins encoded by wnt genes, e.g. Xwnt-8, and wnt receptors to which the glycoproteins bind. In addition, the wnt signal path in man is causally implied in the colon and mammary carcinomas as well as the melanomas (cf. Peifer, M., Science 275, (1997), 1752-1753). Therefore, inhibitors of the wnt signal path could represent a possibility of taking therapeutic against tumoral diseases.

Thus, it is the object of the present invention to provide a product by which the wnt signal path can be inhibited.

According to the invention this is achieved by the subject matters defined in the claims.

Therefore, the subject matter of the present invention relates to an inhibitor protein of the wnt signal path, the protein comprising at least one of the amino acid consensus sequences I and II, indicated in fig. 1.

The present invention is based on the applicant's finding that in animals, particularly mammals, very particularly human beings, there is exists a protein which inhibits the wnt signal path. The applicant has found that *in Xenopus laevis* the expression of the wnt gene, Xwnt-8, results in the formation of Siamese twins. This anomaly will be prevented if the above protein is expressed simultaneously. This protein is

a secretory protein of about 40 kD. It has at least one of the amino acid consensus sequences I and II rich in cysteine and indicated in fig. 1. Variants of the protein are indicated in the form of their DNAs in figure 2. The applicant has also found that variants of the protein are expressed in differing tissues (cf. Table 1 and figure 3).

The present invention refers to the above protein as "wnt inhibitor" (wnt-I).

In a preferred embodiment, (wnt-I) has the amino acid consensus sequences I and II indicated in fig. 1.

A further subject matter of the invention relates to a nucleic acid coding for (wnt-I). It can be an RNA or a DNA. The latter may be a genomic DNA or a cDNA, for example. A DNA is preferred which comprises the following:

the DNA of fig. 2 or a DNA differing therefrom by one or several base pairs,

a DNA hybridizing with the DNA of (a), or

a DNA related to the DNA of (a) or (b) via the degenerated genetic code.

The expression "hybridizing DNA" refers to a DNA which hybridizes with a DNA of (a) under normal conditions, particularly at 20°C below the melting point of the DNA.

The DNA of fig. 2 comprises seven DNAs originating from *Xenopus laevis*, mice, human beings or chickens and coding for (wnt-I). Six of these DNAs were deposited with the DSMZ (*Deutsche Sammlung von Mikroorganismen und Zellkulturen* [German-type collection of micro-organisms and cell cultures]) on September 19, 1997 as follows:

Fig. 2.1 (DNA from human beings) as phdkk-3 under DSM 11762
Fig. 2.2 (DNA from chickens) is termed pcdkk-3
Fig. 2.3 (DNA from mice) as pmdkk-2 under DSM 11759
Fig. 2.4 (DNA from human beings) as phdkk-2 under DSM 11761
Fig. 2.5 (DNA from mice) as pmdkk-1 under DSM 11758
Fig. 2.6 (DNA from human beings) as phdkk-1 under DSM 11760
Fig. 2.7 (DNA from *Xenopus laevis*) as pRNdkk-1 under DSM 11757

A DNA according to the invention is described below in the form of a cDNA. It is exemplary for every DNA falling under the present invention.

For the preparation of a cDNA according to the invention it is favorable to use a *Xenopus laevis* cDNA library as a basis (cf. Glinka, A. et al., Mechanisms Develope 60, (1996), 221-231). Corresponding mRNAs are synthesized from the individual cDNA clones by means of RNA polymerase. They are microinjected into *Xenopus laevis* together with mRNA of wnt genes, e.g. Xwnt-8. *Xenopus laevis* is screened for the development of Siamese twins. The latter are obtained when the mRNA of the wnt gene is microinjected as such or together with such a *Xenopus laevis* RNA which does not code for (wnt-I). Thus, the non-occurrence of Siamese twins is evaluated as an evidence for the presence of an mRNA coding for (wnt-I). Such an mRNA reveals directly the corresponding cDNA.

A cDNA according to the invention can be present in a vector and expression vector, respectively. A person skilled in the art is familiar with examples thereof. In the case of an expression vector for E. coli these are e.g. pGEMEX, pUC derivatives, pGEX-2T, pET3b and pQE-8. For the expression in yeast, e.g. pY100 and Ycpad1 have to be mentioned while e.g. pKCR, pEFBOS, cDM8 and pCEV4 have to be indicated for the expression in animal cells. The baculovirus expression vector pAcSGHisNT-A is especially suitable for the expression in insect cells.

The person skilled in the art knows suitable cells to express a cDNA according to the invention, which is present in an expression vector. Examples of such cells comprise the E. coli strains HB101, DH1, x1776, JM101, JM109, BL21 and SG 13009, the yeast strain *Saccharomyces cerevisiae* and the animal cells L, 3T3, FM3A, CHO, COS, Vero and HeLa as well as the insect cells sf9.

The person skilled in the art knows in which way a cDNA according to the invention has to be inserted in an expression vector. He is also familiar with the fact that this cDNA can be inserted in combination with a DNA coding for another protein and peptide, respectively, so that the cDNA according to the invention can be expressed in the form of a fusion protein.

Furthermore, the person skilled in the art knows conditions of culturing transformed cells and transfected cells, respectively. He is also familiar with processes of isolating and purifying the protein expressed by the cDNA according to the invention. Thus, such a protein, which may also be a fusion protein, also represents a subject matter of the present invention.

A further subject matter of the present invention relates to an antibody directed against an above protein and fusion protein, respectively. Such an antibody can be prepared by common methods. It may be polyclonal and monoclonal, respectively. For its preparation it is favorable to immunize animals - particularly rabbits or chickens for a polyclonal antibody and mice for a monoclonal antibody - with an above (fusion) protein or with fragments thereof. Further "boosters" of the animals may be effected with the same (fusion) protein or with fragments thereof. The polyclonal antibody may then be obtained from the animal serum and egg yolk, respectively. As regards the monoclonal antibody, animal spleen cells are fused with myeloma cells.

The present invention enables to better investigate and understand the wnt signal path. (wnt-I) can be detected in organisms by an antibody according to the invention. In addition, an autoantibody directed against this protein can be detected by a (wnt-I) according to the invention. Both detections can be made by common methods, particularly a Western blot, an ELISA, an immunoprecipitation or by immunofluorescence. Moreover, the expression of the gene coding for (wnt-I) can be detected by a nucleic acid according to the invention, particularly a DNA and primers derived therefrom. This detection can be made as usual, particularly in a Southern blot.

Thus, the present invention also serves for better investigating, i.e. diagnosing, and understanding processes which are connected with the wnt signal path. These are e.g. cell proliferation and differentiation as well as diseases of the most varying kinds. Examples of the latter are diseases of the eyes and bones as well as tumoral diseases, particularly colon and mammary carcinomas as well as melanomas.

Besides, the present invention is suitable to take measures for and against the presence of (wnt-I) in organisms. (wnt-I) can be inhibited in organisms by means of an antibody according to the invention. On the other hand, the amount of (wnt-I) in organisms can be increased by a (wnt-I) according to the invention, particularly after linkage to a protein which is not considered foreign by the body, e.g. transferrin or BSA. The same can also be achieved correspondingly by means of a nucleic acid according to the invention, particularly a DNA, which is controlled by a promoter inducible in certain tissues and which after its expression results in the provision of (wnt-I) in these tissues. In addition, a nucleic acid according to the invention, particularly a DNA, can also be used to inhibit (wnt-I). For this purpose, the nucleic acid is used e.g. as a basis for preparing anti-sense oligonucleotides for the expression inhibition of the gene

coding for (wnt-I).

Thus, the present invention also provides the possibility of interfering with the wnt signal path in an activating fashion and inhibitory fashion, respectively. The former could be made e.g. by administration of an antibody according to the invention against (wnt-I). For the latter, it is an obvious thing to administer (wnt-I) according to the invention. The activation of the wnt signal path could be useful if it is considered to culture organisms for the purpose of organ donation. However, the inhibition of the wnt signal path offers itself so as to be able to take therapeutic steps in the case of diseases of bones and eyes as well as tumoral diseases, particularly colon and mammary carcinomas as well as melanomas.

In particular, the present invention distinguishes itself in that it can be used in tissue-specific fashion. This applies to both diagnosis and treatment. For example, a DNA according to the invention, Dkk-1, a corresponding protein and an antibody thereof, respectively, are particularly suitable for tissues, such as brain, heart, vessels, bones, cartilage, connective tissue and eye. Furthermore, a DNA according to the invention, Dkk-2, a corresponding protein and antibody thereof, respectively, are particularly suitable for tissues, such as brain, heart, vessels, bones, connective tissue, kidneys, testes, spleen, ovaries, muscles, uteri, cartilage, eyes and mammas. Moreover, a DNA according to the invention, Dkk-3, a corresponding protein and an antibody thereof, respectively, are particularly suitable for tissues, such as brain, heart, vessels, bones, cartilage, eyes, connective tissue, lungs, ovaries, muscles and mammas.

Brief description of the drawings:

Fig. 1 shows the amino acid consensus sequences I and II of a (wnt-I) according to the invention. The indication

"-" stands for an amino acid, the number of amino acids being variable when they are provided with an asterisk,

fig. 2 shows the base sequence of seven DNAs coding for (wnt-I) by indicating the bases contributing to the amino acid consensus sequences of (wnt-I),

fig. 3 shows the expression of three DNAs coding for (wnt-I), Dkk-1, Dkk-2 and Dkk-3, in tissues.

The present invention is explained by the below examples.

Example 1: Preparation and purification of a (wnt-I) according to the invention

For the preparation of a (wnt-I) according to the invention, the DNA of fig. 2.6, phdkk-1, was provided with Bam HI linkers, then cleaved by Bam HI and inserted in the expression vector pQE-8 (Diagen) cleaved by Bam HI. The expression plasmid pQ/wnt-I was obtained. Such a plasmid codes for a fusion protein comprising 6 histidine residues (N terminus partner) and a (wnt-I) according to the invention (C terminus partner). pQ/wnt-I was used for transforming E. coli SG 13009 (cf. Gottesman, S. et al., J. Bacteriol. 148, (1981), 265-273). The bacteria were cultured in an LB broth with 100 µg/ml ampicillin and 25 µg/ml kanamycin, and induced with 60 µM isopropyl-ß-D-thiogalactopyranoside (IPTG) for 4 h. Lysis of the bacteria was achieved by the addition of 6 M guanidine hydrochloride. Thereafter, chromatography (Ni-NTA resin) was carried out with the lysate in the presence of 8 M urea in accordance with the instructions from the manufacturer (Diagen company) of the chromatography material. The bound fusion protein was eluted in a buffer having a pH of 3.5 After its neutralization, the fusion protein was subjected to 18 % SDS polyacrylamide gel electrophoresis and dyed with coomassie blue (cf. Thomas, J.O. and Kornberg, R.D., J. Mol. Biol. 149

(1975), 709-733).

It showed that a (fusion) protein according to the invention can be prepared in highly pure form.

Example 2: Preparation and detection of an antibody according to the invention

A fusion protein of Example 1 according to the invention was subjected to 18 % SDS polyacrylamide gel electrophoresis. After dyeing the gel with 4 M sodium acetate, an about 40 kD band was cut out of the gel and incubated in phosphate-buffered salt solution. Gel pieces were sedimented before the protein concentration of the supernatant was determined by SDS polyacrylamide gel electrophoresis which was followed by coomassie blue staining. Animals were immunized with the gel-purified fusion protein as follows:

Immunization protocol for polyclonal antibodies in rabbits

35 μg of gel-purified fusion protein in 0.7 ml PBS and 0.7 ml of complete Freund's adjuvant and incomplete Freund's adjuvant, respectively, were used per immunization.

Day 0: 1st immunization (complete Freund's adjuvant)
Day 14: 2nd immunization (incomplete Freund's adjuvant; icFA)
Day 28: 3rd immunization (icFA)
Day 56: 4th immunization (icFA)
Day 80: bleeding to death.

The rabbit serum was tested in an immunoblot. For this purpose, a fusion protein of Example 1 according to the invention was subjected to SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter (cf. Khyse-Andersen, J., J. Biochem. Biophys. Meth. 10, (1984), 203-209). The Western blot analysis was carried out as

described in Bock, C.-T. et al., Virus Genes 8, (1994), 215-229. For this purpose, the nitrocellulose filter was incubated with a first antibody at 37°C for one hour. This antibody was the rabbit serum (1:10000 in PBS). After several wash steps using PBS, the nitrocellulose filter was incubated with a second antibody. This antibody was an alkaline phosphatase-coupled monoclonal goat anti-rabbit IgG antibody (Dianova company) (1:5000) in PBS. 30 minutes of incubation at 37°C were followed by several wash steps using PBS and then by the alkaline phosphatase detection reaction with developer solution (36 µM 5'-bromo-4-chloro-3-indolyl phosphate, 400 µM nitroblue tetrazolium, 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) at room temperature, until bands were visible.

It showed that polyclonal antibodies according to the invention can be prepared.

Immunization protocol for polyclonal antibodies in chickens

40 µg of gel-purified fusion protein in 0.8 ml PBS and 0.8 ml of complete Freund's adjuvant and incomplete Freund's adjuvant, respectively, were used per immunization.

Day 0: 1st immunization (complete Freund's adjuvant)
Day 28: 2nd immunization (incomplete Freund's adjuvant; icFA)
Day 50: 3rd immunization (icFA)

Antibodies were extracted from egg yolk and tested in a Western blot. Polyclonal antibodies according to the invention were detected.

Immunization protocol for monoclonal antibodies in mice

12 µg of gel-purified fusion protein in 0.25 ml PBS and 0.25 ml of complete Freund's adjuvant and incomplete Freund's

adjuvant, respectively, were used per immunization. The fusion protein was dissolved in 0.5 ml (without adjuvant) in the 4th immunization.

Day 0: 1st immunization (complete Freund's adjuvant)
Day 28: 2nd immunization (incomplete Freund's adjuvant; icFA)
Day 56: 3rd immunization (icFA)
Day 84: 4th immunization (PBS)
Day 87: fusion

Supernatants of hybridomas were tested in a Western blot. Monoclonal antibodies according to the invention were detected.

Table 1: Expression of DNAs according to the invention in mouse embryos

	Dkk-1	Dkk-2	Dkk-3
Neuroepithelium			
E9.5 diencephalon	+++ ventral	+++ medial	+ medial
E12.5	telencephalon M/mantle	hypothalamus	telencephalon M/ventricular zone
Eye	pigmented epithelium	choroid	retina
Spinal cord	-/+	-	ventricular zone Roof plate
Mesoderm:			
Heart E10	bulbis cordis Endocardium septum trans-versum	endothelium	myocardium
Heart E12	endocardial cushion	endothelium	endocard. cushion
Blood vessels	+++ aorta	+++ pulmonary artery	+++ aorta + pulmonary artery
Limbbud mesemchyme	E9 S	I	D
Bone E12	perichondrium	S/mesenchyme	perichondrium I/mesenchyme
Bone E15	Ossification centers	-	-
Urogenital	nephric duct S-shaped body Comma shaped body	metanephric mesenchyme	-
Palate	+++	++	+
Hair follicle	+++ mesenchyme + epithelium	+ -	+ -
Tooth mesenchyme	-	-	+++
Trunk mesoderm	+/-	+++	++

Legend: Mesoderm: (D) deep, (I) intermediate, (L) lateral, (M) medial, (S) superficial
Intensity of expression: (-) absence, (+/-) very weak expression, (+) medium, (++) strong (+++) very strong.